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The intergenic transcribed spacer region 1 as a molecular marker for identification and discrimination of *Enterobacteriaceae* associated with Acute Oak Decline

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1. Abstract

Aims

We assessed the veracity of intergenic spacer region 1 (ITS1) ribotyping for the rapid, inexpensive and accurate identification of *Brenneria goodwinii* and *Gibbsiella quercinecans* that are associated with Acute Oak Decline (AOD) in the UK.

Methods and Results

Agarose gel electrophoresis and polyacrylamide gel electrophoresis (PAGE) were applied for the typing of ITS1 PCR amplicons from strains of *Brenneria goodwinii*, *Gibbsiella quercinecans* and related species (n = 34). The number and length of ITS1 amplicons varied significantly between strains. ITS1 profiles generated via PAGE were used to differentiate species using a neighbour-joining phylogram. The ITS1 phylogram was compared against DNA gyrase B (*gyrB*) gene sequences from the same strains, demonstrating that ITS1 ribotyping is as effective as *gyrB* at resolving *G. qurerciecans* and *B. goodwinii* to the species level.

Conclusions

The ITS1 gene has been successfully employed as a novel marker to resolve newly described AOD associated *Enterobacteriaceae*, *Brenneria goodwinii* and *Gibbsiella quercinecans*, to species level.

Significance and Impact of Study

ITS1 ribotyping of *Brenneria goodwinii* and *Gibbsiella quercinecans* provides equivalent sensitivity to the current standard method for strain identification (sequence analysis of the *gyrB* gene), but with reduced processing time and cost.

Furthermore, the ITS1 gene is widely applicable as a rapid and inexpensive typing system for *Enterobacteriaceae*.

Keywords

Acute Oak Decline, ITS1, DNA gyrase B, *Enterobacteriaceae*, *Gibbsiella quercinecans*, *Brenneria goodwinii*

2. Introduction

Acute Oak Decline (AOD) is a syndrome partly derived from tissue necrosis of the inner bark of oak trees (Denman *et al*. 2014). The decline is relatively new in Britain (reported cases of AOD have been observed for 20-30 years) and has been identified in both species of native oak, with the number of reported cases on the increase. The precise biotic and abiotic causes of AOD are currently unknown, but there are likely to be multiple factors involved. Networks of galleries produced by the larvae of the buprestid beetle *Agrilus biguttatus* have been discovered in trees with symptoms of AOD and are typically associated in and around areas of lesion formation (Denman *et al*. 2014). The beetles themselves are rarely seen, but evidence of their occurrence is presented via larval galleries in the cambial zone of the oak tree. In addition, previous studies have described the consistent isolation of *Enterobacteriaceae* from necrotic lesions of affected oak trees in sampling sites across England (Brady *et al*. 2010; Denman *et al*. 2012). However, two newly-described bacterial species belonging to the *Enterobacteriaceae*, *Gibbsiella quercinecans* and *Brenneria goodwinii*, are consistently isolated from necrotic lesions on oak trees, with other species such as *Rahnella* spp. isolated from both

healthy and necrotic tissues. Consequently, the almost exclusive association of *G. quercinecans* and *B. goodwinii* with the lesions of AOD affected trees has led to the hypothesis that these species play a central role in tissue necrosis (Denman and Webber 2009), possibly via necrogenic enzymes or secretion systems and their associated effector proteins.

The ribosomal RNA (rrn) operon and in particular, 16S rRNA, are conserved genomic regions commonly used for bacterial genotyping and taxonomy (Woese and Fox 1977; Cedergren *et al*. 1988). However, due to the highly conserved nature of the 16S rRNA gene, resolution of closely related species within certain taxa such as the *Enterobacteriaceae* can be problematic (Janda and Abbott 2007; Naum *et al*. 2008). The dearth of sequence differences among closely related species contributes to a lack of phylogenetic power and limits its applicability for fine scale taxonomic resolution. The intergenic or internal transcribed spacer region 1 (ITS1) is part of the *rrn* operon, found between the genes for small (16/18S) and large (23/28S) rRNA subunits. The ITS1 region is more susceptible to synonymous mutations than other parts of the operon due to its functional role being confined to the coding of tRNAs and processing rRNA (Scheinert *et al*. 1996). Consequently, it is an excellent candidate for molecular differentiation between both diverse and closely related species. ITS analysis, sometimes called RISA (Ribosomal Intergenic Spacer Analysis) is a method most commonly applied to examine the extent of microbial diversity in environmental samples from soil and marine communities e.g. Brown and Fuhrman (2005), Rappe *et al.* (2006), and it has been well described in medical microbiology for identifying species in clinical samples e.g. Gurtler and Stanisich (1999). However, its use in arboreal microbial analysis has not previously been tested. Molecular studies typically use the 16S rRNA gene for taxonomic

identification at the familial level, and although this is the gold standard of PCR based identification of taxonomic identity, it often lacks accuracy in discriminating at the species level (Gurtler and Stanisich 1999). Conversely, the ITS1 region is highly variable in length and sequence in all bacteria and may thus be effective at distinguishing bacterial species and strains. It should be noted that many bacteria contain more than one copy of the *rrn* operon, which usually show strong sequence homogeneity (>98%) but may differ through insertions or deletions by a range of 2-301 bp, with an average difference of 166 bp (Klappenbach *et al*. 2001; Larkin *et al*. 2007). ITS1 amplicons will therefore exhibit substantial variation in size as amplicon range can vary from 150-1500 bp with 85-90% of amplicons being between 150-600 bp (Fisher and Triplett 1999). Often the ITS1 region will code for tRNA genes, these are most frequently found in Gram-negative bacteria and give extra length to the sequence. However if present they are usually more conserved than other parts of ITS1 (Daffonchio *et al*. 1998). Typically, the ITS1 amplicon pattern resolved using agarose gels can be used to identify species, whereas the hypervariable indels (mutations) can be used to differentiate between strains (Garcia-Martinez *et al*.1999).

Due to the conserved nature of 16S rRNA genes across the *Enterobacteriaceae*, *gyrB* is used as the phylogenetic marker gene of choice for identification and taxonomic resolution of strains isolated from the necrotic lesions of oaks affected by AOD. This approach has provided important insights into the ecology and epidemiology of *G. quercinecans* and *B. goodwinii* and their almost exclusive association with necrotic tissue in AOD affected trees. However, *gyrB* gene PCR amplification, sequencing and phylogenetic analysis of hundreds/thousands of isolated strains from affected trees is a costly and laborious process, representing a

barrier to elucidating the ecology and epidemiology of *B. goodwinii* and *G. quercinecans.* This is a pertinent issue, as there are numerous ongoing investigations into the role of bacteria in AOD and the need to confirm the identity and purity of strains is of paramount importance.

The aim of this study was therefore, to develop a rapid molecular diagnostic test for *B. goodwinii* and *G. quercinecans*, based on ITS1 profiling, to resolve species identity and purity of strains. We hypothesised that the ITS1 region represents a suitable genotypic marker for identification and differentiation of *B. goodwinii* and *G. quercinecans* strains associated with AOD and negates the requirement for gene sequencing, as multiple copies of the ITS1 region are present in the bacterial genome, with significant size variation in the length of the sequence.

Here, the ITS1 profiles of bacterial isolates from the necrotic lesions of oak trees were characterised and validated using a polyphasic analysis. Two DNA fingerprinting methods were tested and compared to verify and validate the use of ITS1 profiles in species identification and resolution. Amplified ITS1 PCR products were resolved via (i) 3% agarose gel electrophoresis and (ii) polyacrylamide gel electrophoresis. Finally, the ability to resolve isolated strains to species level was determined by a comparative phylogram of ITS1 profiles and the DNA gyrase B gene sequence of each strain.

3. Methods

3.1 Maintenance of bacterial strains

Enterobacteriaceae strains were isolated by Forest Research, from oak trees affected by AOD. Most strains had been previously identified to species level through DNA gyrase B (*gyrB*) sequencing and DNA-DNA hybridisation (Brady *et al*. 2010; Denman *et al*. 2012). The strains were stored in glycerol stocks at -80°C and maintained on nutrient agar (Oxoid) at 20°C.

3.2 ITS1 PCR amplification, agarose gel electrophoresis and sequencing

Each strain was sub-cultured from an individual colony five times, creating five sub-isolates per strain. Genomic DNA was prepared using the colony extraction method for ITS1 PCR reactions: one colony of bacterial cells was picked from an agar plate and added directly to the PCR assay tube (colony PCR). The ITS1 region of each sub-isolate was amplified using ITS1 specific oligonucleotide primers, designed for environmental bacterial communities ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale *et al*. 2004). PCR reactions were performed in 50 µL reaction volumes containing; a colony of each isolate, 1 x MyTaq Red Mix (Bioline), 50 µmol l^{-1} each forward and reverse primer. The solution was made up to its final volume of 50 µL with molecular grade double distilled water. The thermal cycle consisted of initial denaturation at 95°C for 60s followed by 25 cycles of denaturation at 95°C for 15s, primer annealing at 55°C for 15s and elongation at 72°C for 10s. PCR amplification products were visualised using 3% agarose gel at 120 V for 135 minutes and amplicon size was calculated using Hyperladder I (Bioline). ITS1 amplicons were excised from agarose gels and extracted directly using the QIAEX II get extraction kit (Qiagen) for all sequencing

reactions. Purified PCR amplicons were sequenced by Macrogen Inc. These sequence data have been deposited to Genbank under accession numbers KJ418748 to KJ418834.

3.3 *gyrB* PCR amplification, gel electrophoresis and sequencing

The type II topoisomerase *gyrB* gene was selected as a quality control marker to positively identify bacterial species and verify the utility of ITS1 as a molecular marker for identification and discrimination of bacterial species. Oligonucleotide primers were *Enterobacteriaceae* specific as described by Brady *et al*. (2010), gyrB01F (5'-TAARTTYGAYGAYAACTCYTAYAAAGT-3') and gyrB02R (5'-CMCCYTCCACCARGTAMAGT-3'). A separate forward primer was used for sequencing of *gyrB* PCR amplicons, gyrB07F (5'-GTVCGTTTCTGGCCVAG-3'). Genomic DNA was prepared using the boil prep method, where a colony of the isolate was picked from nutrient agar plates and suspended in 20 µl molecular grade water and incubated at 100°C for 5 mins before adding 1 µl to the PCR reaction mixture. PCR reactions were performed as described for ITS1 with an annealing temperature of 50°C. PCR amplification products were visualised via 1% agarose gel electrophoresis at 140 V for 35 minutes. *gyrB* amplicons were excised from the gel and purified using the QIAEX II kit (Qiagen). Purified amplicons were sequenced at Macrogen Inc.

3.4 Polyacrylamide Gel Electrophoresis (PAGE)

The ITS1 region of thirty-four bacterial isolates from AOD affected trees was amplified as described for the agarose gel analysis. Electrophoresis was conducted using an Ingeny PhorU electrophoresis unit (Ingeny, Leiden, Netherlands). ITS1 PCR products (50 µl, prepared as described in section 3.2) were loaded into a 15%

polyacrylamide gel and allowed to migrate through the gel for 16 hours at 100 V. Amplicons were then visualised using a SYBR Gold stain (Invitrogen).

3.6 Phylogram analyses of ITS1 amplicon patterns and *gyrB* DNA sequences

Phylogenetic analyses of all thirty-four AOD isolates based on separation of their ITS1 amplicons from the PAGE analysis was conducted in Dendroscope (Huson *et al*. 2012). The size (in base pairs) of ITS1 amplicons were scored individually using Bio-Rad Chemidoc software, which aligned matching amplicons in a presence absence test (with a sensitivity setting of 2) giving a binary output. The binary output was then altered; changing '0' to 'T' and '1' to 'C'. This altered binary data was entered into ClustalW (Larkin *et al*. 2007), which produced a neighbour joining phylogenetic tree based on presence and absence of amplicons. The resulting tree was entered into Dendroscope and modified into both a rectangular phylogram and a tanglegram. The phylogram was used to display ITS1 fingerprints from the polyacrylamide gel. The tanglegram directly compared the PAGE analysis against DNA gyrase B gene sequences for thirty-three AOD strains.

4. Results

4.1 Validation of ITS1 typing as a tool for species resolution

Six bacterial strains (two x *G. quercinecans*, two x *B. goodwinii* and two x *Rahnella* spp.) were used to validate the veracity of the ITS1 amplicon typing approach. In order to confirm the purity of these strains, each strain was consecutively plated from a single colony five times and this subset (n=30 sub-isolates) was characterised by

ITS1 PCR amplicon typing and *gyrB* gene sequencing to confirm that each isolate was correctly identified (Table S1).

ITS1 specific PCR amplicons were generated for each of the thirty sub-isolates and resolved via 3% agarose gel electrophoresis (e.g. Fig. 1). The number of visible ITS1 amplicons for each strain tested varied from three to seven, with both strains of *G. quercinecans* possessing six ITS1 amplicon bands. The two *B. goodwinii* strains possessed different numbers of visible ITS1 amplicons (four and six respectively) and *Rahnella* sp. strains also possessed a different number of amplicons (four and seven) (Table S1). These data demonstrate that ITS1 typing provides an effective method for the resolution of the six different *Enterobacteriaceae* strains tested at the species level. Furthermore, despite the presence of six amplicons for both *G. quercinecans* strains tested, differences in the length of these ITS1 amplicons provided strain level differentiation, and this was also the case for *B. goodwinii* and *Rahnella* sp. strains, which demonstrated the potential for strain specific resolution, where both the number and length of ITS1 amplicon varied between the two strains of each species.

Visible ITS1 amplicons for all sub-isolates were subsequently excised and purified from agarose gels, and where sufficient DNA was retrieved, each amplicon was sequenced to confirm its identity. ITS1 amplicon sequencing (n=87) and BLASTn searches (Altschul *et al*. 1990) confirmed that all observed ITS1 amplification products were *bona fide* ITS1 sequences. In addition, each strain was identified via a confirmatory *gyrB* sequencing reaction (Table S1). However, for one of the strains (*G. quercinecans* FRB98) agarose gel electrophoresis of five sub-isolates obtained from the same agar culture plate revealed two distinct ITS1 amplicon types (Fig. 1), where the presence of a contaminant is clearly visible, as three sub-isolates have

three visible amplicons and two isolates have six amplicons. These data suggested the presence of a second bacterial strain in the culture of *G. quercinecans* FRB98 and *gyrB* sequencing confirmed the presence of two strains within the culture; *G. quercinecans* FRB98 (lanes 5 and 6, Fig. 1) and *Enterobacter cloacae* (lanes 2-4, Fig. 1), demonstrating an additional application of ITS1 ribotyping in strain maintenance and the identification of culture contaminants in laboratory strains. The pilot study therefore validated the use of ITS1 typing to separate members of the *Enterobacteriaceae* at both species and strain level, and this approach was subsequently tested and validated on three commonly used and independent laboratory methods for DNA fingerprinting.

4.2 Comparison of three independent methods for the resolution of ITS1 ribotypes in *Enterobacteriaceae* strains associated with AOD

4.2.1 3% agarose gel electrophoresis

Based on the results of the pilot study the ITS1 genomic region was proposed as an appropriate molecular marker for the resolution of members of the *Enterobacteriaceae*. The agarose verification method was subsequently extended to a wider selection of *Enterobacteriaceae* strains (n=34) (Table S2). However, variations in ITS1 amplicon numbers for some strains were observed when the same strain was run on replicate agarose gels despite identical quantities of PCR amplified ITS1 fragments being loaded onto the gel. It is possible that in independent PCR reactions, certain amplicons are preferentially amplified due to PCR bias and agarose is not as sensitive a tool to visualise DNA fragments. Nevertheless, the sensitivity of detection using 3% agarose gel separation of ITS1 fragments did appear to provide a rudimentary resolution of strains and species, but the ITS1

amplicon differences observed between the same strain run on replicate agarose gels suggest that alternative and more sensitive method for ITS1 typing would be more appropriate. Consequently, polyacrylamide gel electrophoresis analysis of ITS1 PCR products was tested as an alternative method for the resolution of ITS1 ribotypes and compared with phylogenetic resolution against the *gyrB* gene phylogeny for each strain.

4.2.2 PAGE resolution of *Enterobacteriaceae* ITS1 ribotypes

The ITS1 ribotype profiles of the same thirty-four *Enterobacteriaceae* strains tested via agarose gel electrophoresis were analysed using PAGE to further test the utility of the ITS1 region as a marker for strain discrimination and provide greater resolution of the ITS1 amplicons (Fig. 2).

Visual inspection of ITS1 profiles for *G. quercinecans* and *B. goodwinii* (Fig. 2), suggests similarities between the ITS1 ribotypes of isolates of the same species. This discrimination was further demonstrated using a neighbour joining phylogenetic tree to infer relationships between species (Mount, 2008).

Phylogenetic resolution of the thirty-four AOD isolates based on their ITS1 profile using PAGE successfully separated *G. quercinecans* and *B. goodwinii* strains into two lineages (Fig. 3). Twenty of the 34 strains were *Gibbsiella quercinecans* and *Brenneria goodwinii* isolates, and to further validate the use of ITS1 as a molecular marker for the discrimination of *G. quercinecans* and *B. goodwinii*, a broad spectrum of related AOD strains (n=14) from the *Enterobacteriaceae* were also separated and compared using ITS1 ribotyping.

An amplicon presence/absence matching alignment was used to generate the phylogenetic tree in Fig. 3, to demonstrate the relationship between isolates. One potential caveat to this approach is that similarly sized ITS1 amplicons can erroneously be grouped together, as the QuantityOne software (Bio-Rad) used in this study (or the similar amplicon matching open source software PyElph 1.4 (Pavel and Vasile 2012)), cluster amplicons of a similar but not identical size. However, our data suggest that this does not result in the mis-identification of strains at the species level at least. These programs do have an adjustable sensitivity gauge, but ultimately they must be manually validated. An appropriately spaced molecular ladder (i.e. a long gap between each rung) can reduce bias by creating a larger area on the gel image.

The PAGE derived ITS1 phylograms were compared against *gyrB* sequences using a tanglegram (Fig. 4). This contrasting phylogram reveals the differences in resolution between *gyrB* sequencing and ITS1 ribotyping methods, but demonstrates the ability of both methods to resolve *G. quercinecans* and *B. goodwinii* at the species level. Furthermore, some strains could be resolved through their ITS1 amplicon pattern, for example *G. quercinecans* FRB92 has five amplicons whereas *G. quercinecans* FRB93 has four. PAGE successfully resolved ITS1 ribotypes to the species level for all *G. quercinecans* and *B. goodwinii* isolates, barring one exception; *Gibbsiella gregii* USA42 clustered with *G. quercinecans* FRB185 and the latter strain has therefore not been successfully resolved according to species. However, it is clear from the PAGE profiles that *G. quercinecans* strain FRB185 has a different ITS1 PAGE profile to both *G. gregii* USA42 and also the other members of that species, but as it is currently a singleton strain for this ITS1 ribotype, it appears to be an outlier. Furthermore, *G. quercinecans* strain FRB185 also failed to cluster

with the other members of this species via *gyrB* sequence analysis (Fig. 4), and clustered with *Gibbsiella dentisursi* and *G. papillionis*, suggesting that the inability of both ITS1 ribotyping and *gyrB* sequencing to resolve FRB185 to species level is an issue specific to that strain, rather than a methodological issue. Nevertheless, *G. quercinecans* FRB185 occupies the same clade as the other *G. quercinecans* strains and it is likely that future typing of additional *G. quercinecans* strains will further resolve the intra-species and genus relationships between strains.

Discrimination of closely related bacterial strains using ITS1 amplicon length may sometimes be challenging as it is not possible to ensure each copy of the ITS1 region is amplified with equal concentration. Therefore some ITS1 copies which may be present in the genome may not appear when visualised on a gel image or appear as a shadow. It should be noted that it is possible if not probable that not all ITS1 copies are resolved. However, resolved ITS1 fragments were visualised using PAGE consistently and reproducibly and the method was capable of validation of the ITS1 maker and separation of the AOD isolates. On a cautionary note, there may be similarly weighted ITS1 fragments shared between unrelated bacteria causing relatedness between bacteria to be inferred when no such genotypic relationship exits (Jensen *et al*.1993). This is likely to be a rare event, but it should be noted as overlapping intergenic spacer size classes could lead to misinterpretation of results.

4.2.4 *gyrB* phylograms vs ITS1 typing

16S rRNA gene sequencing represents the most commonly used phylogenetic tool for estimating microbial diversity and would be improperly applied to the *Enterobacteriaceae* here due to the conserved nature of the phylum at this locus (Mollet *et al*. 1997). Therefore, to resolve interspecies taxonomic relatedness the

gyrB gene is the marker of choice for taxonomic identification of *Enterobacteriaceae* strains isolated from AOD affected trees (Brady *et al*. 2010; Denman *et al*. 2012). Relationships between *Enterobacteriaceae* strains were revealed using the *gyrB* molecular marker to create a neighbour joining topology. This topology was compared to a neighbour joining phylogram from the polyacrylamide gel generated via the ITS1 molecular marker using a tanglegram (Fig. 4). The tanglegram reveals that species level resolution of ITS1 is very similar to that of *gyrB*. Furthermore strain separation may be possible using ITS1, especially in strains with a higher number of ITS1 copies, as this gives a greater prospect of variation. For example, *G. quercinecans* has fewer ITS1 copies than *B. goodwinii*, and this increases the phylogenetic resolution of ITS1 in *B. goodwinii* compared to *G. quercinecans*. Additionally, as Fig. 4 reveals, *B. goodwinii* FRB171 and *B. goodwinii* PFK1/3B are identical in their *gyrB* sequence, whereas the ITS1 copy number differs, and they are therefore separated into different branches of the tree. Therefore, as ITS1 copy number increases, the marker contains ever greater phylogenetic power to differentiate between closely related strains to a greater extent than that of *gyrB*. The tanglegram analysis conclusively demonstrates the suitability of ITS1 ribotyping for the resolution of *Enterobacteriaceae* strains.

5. Discussion

Gibbsiella quercinecans and *Brenneria goodwinii,* belonging to the family *Enterobacteriaceae,* are strongly suspected to play a leading role in AOD, but one of the difficulties faced by microbiologists working in the field is culture-based differentiation of AOD bacterial isolates, which are morphologically similar and

require strain purification, DNA extraction, PCR amplification of the *gyrB* gene, sequencing and phylogenetic analysis for taxonomic identification. This study demonstrates that ITS1 profiling can discriminate between species amongst the family *Enterobacteriaceae* to the same resolution as the most commonly used differentiation marker, DNA gyrase B (*gyrB*). The advantage that ITS1 ribotyping has over *gyrB*, is that it negates the requirement for a sequencing reaction and DNA sequence analysis, therefore reducing both cost and time. Polyacrylamide gel electrophoresis successfully separated ITS1 amplicons using intergenic heterogeneity in the ITS1 region using standard protocols. *gyrB* sequencing of individual AOD bacterial isolates validated the ITS1 amplicon separation and phylogenetic analyses providing evidence that it could be used as a relatively rapid and inexpensive environmental bacterial identification method (Fig. 4).

Beyond the successful resolution of the two species of interest (*Gibbsiella quercinecans* and *Brenneria goodwinii*), our data suggest that ITS1 ribotyping analysis is not suitable for the discrimination of genus-level relationships. As described above, low fidelity of the ITS1 region provides the basis for this study. However, variability of the marker is such that inter-genus relationships are idiosyncratic and meaningful comparisons are limited. This is demonstrated via Figs. 3 and 4 where the *Gibbsiella quercinecans* and *Brenneria goodwinii* species are robustly separated into distinct clades. However, drawing any phylogenetic conclusions between genera for the AOD isolates used in this study, is not supported via bootstrapping (Fig. 3). For example, on the PAGE phylogenetic tree, *Rahnella sp.*1 USA39 and *Brenneria salicis* DSM30166 are placed on the same phylogenetic node, yet based on the random sampling of the bootstrapping analysis this node is not strongly supported, hence it could not be inferred that these isolates are closer

than other inter-genus relationships on the tree. Therefore our data has shown similar to previous studies (Gurtler and Stanisich 1999), that ITS1 copy numbers are strain specific with inter-genus level relationships unclear.

In this study, ITS1 ribotyping has been tested and validated on arboreal *Enterobacteriaceae,* using two independent electrophoresis methods. However, it could equally be applied to other members of the *Enterobacteriaceae* including bacterial groups in clinical studies. There are reports in the literature on the inefficiency of the 16S rRNA gene or other conventional identification methods for screening bacterial isolates due to the sequence similarity between closely related species (Fukushima *et al*. 2002; Paradis *et al*. 2005; Pavlovic *et al*. 2011). For example, *Escherichia coli* and *Shigella* spp. pathovars are closely related with few disparate biochemical characteristics. Consequently, separation using biochemical tests or the 16S rRNA gene will often fail to resolve strains. Therefore, the method we propose here could thus be applied to other situations. Equally, the food industry requires diagnostic mechanisms for *Enterobacteriaceae* species, as these through a variety of species or strain specific mechanisms can spoil meat products (Doulgeraki *et al*. 2011; Mofokeng *et al*. 2011). Through building ITS1 profiles for individual *Enterobacteriaceae* species the method offers a rapid fingerprinting mechanism without the need for expensive and time consuming sequencing reactions, which is not only applicable in arboreal studies but across all disciplines of microbiology.

This is a simple, accurate, and inexpensive method for the rapid screening of *Enterobacteriaceae* samples and provides robust species specific profiles. The

method allows environmental screening of a large number of samples providing species identification and differentiation data.

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Conflict of interest

No conflict of interest declared

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Figure 1 Five sub-isolates (lanes 2-6) from a culture of *G. quercinecans* strain FRB98 revealed two distinct ITS1 amplicon patterns on a high resolution 3% agarose gel, suggesting that the culture was impure. *gyrB* sequencing of sub-isolates in lanes 5 and 6 confirmed that these sub-isolates were *G. quercinecans*, whereas *gyrB* sequencing of sub-isolates in lanes 2-4 established that the sub-isolates were *Enterobacter cloacae*. Lane 1 contains Hyperladder I (Bioline). Ladder size is given in base pairs.

Figure 2 Polyacylamide gel with ITS1 profiles for: *Brenneria goodwinii*, lanes 2- 6, 8-9, 11-13; *Gibbsiella quercinecans*, lanes 14-15, 17-21, 23-25. Lanes 1,7,10,16 and 22 contain Hyperladder I (Bioline). Blank lanes are not included in the lane numbering. Ladder size is given in base pairs.

Figure 3 Neighbour-joining phylogram of AOD isolates and their ITS1 fingerprints resolved on a polyacrylamide gel. Nodes in which bootstrap value >95% are denoted as filled circles and those between 75% and 95% as unfilled circles.

Figure 4 Tanglegram comparing *gyrB* phylogram (left) and polyacrylamide phylogram (right). GenBank accession numbers are given in parentheses. Bootstrap values >95% are denoted as filled circles, values between 75% and 95% are denoted as clear circles.